

Genetic differentiation of *Culex pipiens* (Diptera: Culicidae) in China

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Abstract

The population genetic structures of *Culex pipiens* Linnaeus were evaluated in China over a 2000 km transect that encompasses the two subspecies, *C. p. pallens* and *C. p. quinquefasciatus*. Four polymorphic allozyme loci were investigated in 1376 mosquitoes sampled from 20 populations across four provinces. These loci were not statistically dependent with no apparent heterozygote deficit or excess. On a regional scale (intra-province), a low ($F_{st}=0.007-0.016$) and significant genetic differentiation was found, with no clear geographical pattern. On a wider scale (inter-province), the genetic differentiation was higher ($F_{st}=0.059$), and an isolation by distance emerged. The results are compared with previous population genetic surveys of this mosquito species in different geographic areas over the world. The overall pattern suggests that *Culex pipiens* requires considerable distance (500–1000 km) to show isolation by distance, irrespective of the subspecies (*C. p. pipiens*, *C. p. quinquefasciatus* and *C. p. pallens*) or the geographic location.

Keywords: *Culex pipiens*, population genetics, genetic differentiation, distance isolation

Introduction

The mosquito, *Culex pipiens* Linnaeus (Diptera: Culicidae), is a ubiquitous species that colonizes a large variety of biotopes throughout temperate and tropical countries. It is usually considered as a complex including: *C. p. pipiens* Linnaeus, *C. p. quinquefasciatus* Say, *C. p. pallens* Coquillett and *C. p. molestus* Forskål (Knight & Stone, 1977; Knight, 1978). These are morphologically, physiologically, ecologically and behaviourally different. *Culex p. pipiens* (temperate type) and *C. p. quinquefasciatus* (tropical type) are the most widely found members and are both closely associated with human activity. They are major vectors of *Wuchereria bancrofti* and West Nile virus (Anderson *et al.*, 1999). There

has been much research on *C. pipiens* throughout the world on various subjects, such as insecticide resistance and population genetic structure. Understanding the population structure of the mosquito may provide valuable information about its migration pattern, which is essential for assessing the spread of selected traits such as insecticide resistant genes (Lenormand & Raymond, 1998).

All four subspecies from the *C. pipiens* complex have been found in China (Zhao & Lu, 1995; Qu, 1999). Unlike for the western and central Palaearctic, where *C. p. pipiens* is the most common type, *C. p. pallens* is the dominant subspecies in northern China, whereas *C. p. pipiens* is only found in the Xingjiang Uygur Autonomous Region. *Culex p. quinquefasciatus* is found mainly in southern China and *C. p. molestus* has so far been detected only around Beijing (Zhao & Lu, 1995). Hybridization tests showed that there were no reproductive isolations between *C. p. pipiens*, *C. p. pallens* and *C. p. quinquefasciatus*, and there are some overlapping areas between the distributions of *C. p. pipiens* and

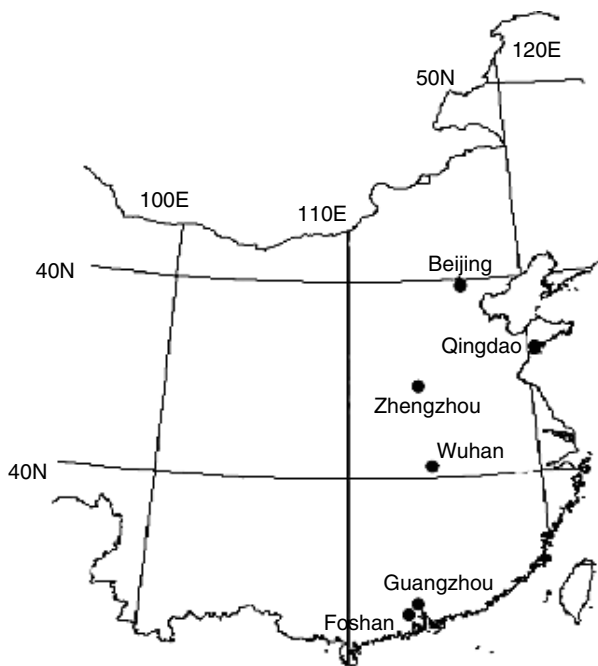
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Table 1. Collection sites of *Culex pipiens* sampled in China in 2003.

Province or municipality	Locality (latitude, longitude)	Code	No.	Date (d/m)	Type of site
Guangdong	Guangzhou (23°08'N, 113°15'E)	Karaoke	1	28/09	Sewage puddle
		Jin1	2	28/09	Sewage tank
		Jin2	3	28/09	Well
		Lin	4	28/09	Sewage
		GongDi	5	27/09	Cesspool
	Foshan (23°03'N, 113°06'E)	Manda1	6	30/09	Puddle
		Manda2	7	30/09	Puddle
		Manda3	8	30/09	Puddle
		Manda4	9	30/09	Puddle
Hubei	Wuhan (30°37'N, 114°21'E)	ZhuChang	10	24/09	Cesspool
		CaiYuan	11	23/09	Ditch
		LanXi	12	23/09	Vat
Henan	Zhengzhou (34°48'N, 113°42'E)	Shen	13	19/09	Water tank
		TaiQiu	14	19/09	Ditch
Shandong	Qingdao (36°04'N, 120°18'E)	QingDao	15	15/07	River puddle
Beijing	Beijing (39°54'N, 116°28'E)	BJBJT	16	10/09	Sewage
		BJTJL	17	10/09	Ditch
		BJSGZ	18	11/09	Cesspool
		BJFT	19	11/09	Sewage
		BJHY	20	11/09	Sewage

Fig. 1. Locations of population collections of *Culex pipiens* complex in China in 2003.

C. p. pallens, *C. p. pallens* and *C. p. quinquefasciatus* (Zhao & Lu, 1996). Biosystematic studies (including hybridization), morphological studies and gas chromatography analyses of cuticular hydrocarbons confirmed *C. p. quinquefasciatus* and *C. p. pallens* as being subspecies in this complex (Zhao & Lu, 1995). The present study aimed to investigate the population genetic structure of the *C. pipiens* complex (*C. p. quinquefasciatus* and *C. p. pallens*) in China. Sampling was carried out

at different scales over a south–north transect across the country, allowing us to determine genetic differentiation and isolation by distance.

Materials and methods

Mosquitoes

Larvae and pupae of *C. pipiens* were collected during 2003 from four provinces and one municipality (Beijing), from a total of 20 breeding sites (fig. 1, table 1). Whenever possible, every mosquito was raised until its adult stage and then deep frozen until used for enzyme characterization.

Electrophoresis

Using starch gel electrophoresis (TME 7.4 buffer system) of adult mosquito homogenates (Pasteur *et al.*, 1988), we studied electrophoretic polymorphisms of the following four enzymes: glutamate-oxaloacetate transaminase (EC 2.6.1.1), hexokinase (EC 2.7.1.1), phosphogluco-isomerase (EC 5.3.1.9), and phosphoglucomutase (EC 2.7.5.1). Two strains, derived from S-LAB (Georghiou, 1966) and having the same genetic background were used as reference standards: SA2 and SB1 (Berticat *et al.*, 2002). For each enzyme, the electromorph band for the reference strain was designated '100' and other electromorph bands (alleles) were numbered according to their relative electrophoretic mobility, that is 110, 120, etc. for faster bands and 90, 80, etc. for slower bands.

Data analysis

Each locus was tested for conformity with Hardy-Weinberg (HW) equilibrium using the exact U-score test with heterozygote deficiency being the alternative hypothesis (Rousset & Raymond, 1995). A global test across samples and/or loci was also carried out (Rousset & Raymond, 1995).

Table 2. Allelic frequencies observed at four putative allozyme loci for 20 mosquito populations. Population numbers are given in table 1.

Loci	Populations																				All
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
GOT-2	(62)	(62)	(91)	(62)	(19)	(69)	(27)	(62)	(62)	(62)	(80)	(64)	(102)	(92)	(73)	(89)	(62)	(62)	(62)	(89)	
60	0	0	0.01	0	0	0	0	0.02	0	0.09	0.02	0	0	0.03	0	0	0	0	0	0	0
70	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.03	0	0	0	0	0
80	0.56	0.65	0.60	0.56	0.47	0.62	0.74	0.73	0.57	0.23	0.38	0.45	0.34	0.42	0.28	0.38	0.31	0.30	0.35	0.33	
90	0.02	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0.09	0	0	0	
100	0.43	0.34	0.38	0.44	0.53	0.38	0.26	0.23	0.43	0.68	0.61	0.55	0.66	0.55	0.72	0.59	0.60	0.69	0.65	0.66	
120	0	0.02	0.01	0	0	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0.01	
140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01	0	0	
Fis	+0.15	+0.18	+0.14	-0.04	+0.18	-0.10	+0.05	-0.02	-0.08	+0.10	-0.04	+0.22	+0.22	-0.17	+0.16	+0.01	-0.10	+0.26	-0.19	+0.21	+0.05
PGM	(62)	(62)	(91)	(62)	(19)	(39)	(27)	(62)	(62)	(62)	(78)	(64)	(82)	(92)	(69)	(89)	(61)	(62)	(62)	(89)	
60	0	0	0	0	0	0	0	0	0	0	0	0.01	0.01	0.01	0.01	0	0	0.01	0	0	0
80	0.03	0.10	0.05	0.10	0.05	0.09	0.04	0.10	0.05	0.21	0.17	0.32	0.21	0.28	0.28	0.29	0.25	0.32	0.24	0.31	
90	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01	0.01	0.01	0	0	0	0	
100	0.80	0.81	0.87	0.81	0.79	0.83	0.85	0.74	0.86	0.70	0.70	0.59	0.66	0.53	0.57	0.58	0.62	0.56	0.60	0.59	
110	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01	0	0	0	0	
120	0.17	0.08	0.08	0.08	0.16	0.08	0.11	0.16	0.09	0.09	0.13	0.08	0.12	0.18	0.12	0.11	0.13	0.10	0.15	0.10	
140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01	0	0	0	0	0	
Fis	-0.20	-0.05	+0.08	-0.10	-0.03	-0.13	-0.12	-0.16	+0.02	+0.05	-0.10	+0.25	+0.14	-0.08	-0.07	-0.08	-0.19	+0.04	-0.04	+0.02	-0.03
PGI	(62)	(62)	(91)	(62)	(19)	(69)	(27)	(62)	(62)	(62)	(80)	(85)	(102)	(92)	(69)	(90)	(61)	(62)	(62)	(59)	
80	0.01	0	0	0.01	0	0	0	0.02	0	0	0	0	0	0	0	0.01	0	0.01	0	0.02	0
90	0	0.02	0.01	0	0	0.05	0	0.01	0.02	0.10	0.06	0.06	0.05	0.04	0.14	0.07	0.22	0.06	0.08	0.06	
100	0.99	0.98	0.99	0.99	1.00	0.94	1.00	0.97	0.98	0.90	0.94	0.94	0.95	0.95	0.86	0.93	0.77	0.94	0.89	0.94	
110	0	0	0	0	0	0.01	0	0.01	0.01	0	0	0.01	0	0.01	0	0.01	0	0	0.01	0	
Fis	-	-0.02	-0.01	-	-	-0.05	-	-0.01	-0.01	-0.10	+0.18	-0.06	-0.05	+0.19	-0.16	-0.07	-0.28	-0.05	+0.07	-0.05	-0.06
HK	(62)	(62)	(61)	(62)	(19)	(69)	(27)	(62)	(62)	(62)	(80)	(70)	(84)	(63)	(74)	(61)	(62)	(62)	(62)	(89)	
90	0	0	0.01	0.01	0.03	0	0	0	0.01	0	0	0	0	0	0.07	0	0.05	0	0	0	
100	1.00	0.99	0.99	0.99	0.97	0.98	1.00	1.00	0.98	0.98	1.00	1.00	0.98	1.00	0.91	0.98	0.90	0.95	0.98	0.98	
105	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
110	0	0.01	0	0	0	0.02	0	0	0.01	0.02	0	0	0.02	0	0.02	0.02	0.05	0.05	0.02	0.02	
Fis	-	-	-	-	-	-0.01	-	-	-0.004	-0.01	-	-	-0.02	-	-0.08	-0.01	-0.07	-0.04	-0.02	-0.01	-0.04

Genetic differentiation of mosquitoes

The number of mosquitoes analyzed is given in parentheses. Bold Fis values indicate a significant ($P < 0.05$) departure from HW due to heterozygote deficiency.

Table 3. Differentiation (F_{st}) among populations of *Culex pipiens* in China.

Locus	Overall	Subdivision				
		Intra province				Inter province
		Guangdong	Beijing	Hubei	Henan	
Got-2	0.076	0.014	0.003	0.025	0.012	0.082
PGM	0.047	0.006	-0.003	0.013	0.017	0.056
PGI	0.041	0.008	0.045	0.0001	-0.005	0.026
HK	0.027	0.001	0.017	0.010	0.014	0.024
All	0.059	0.011	0.007	0.016	0.013	0.063

'Overall' refers to the estimate across all populations. 'All' refers to the multi-locus estimate. Bold characters indicate significant ($P < 0.05$) genotypic differentiation.

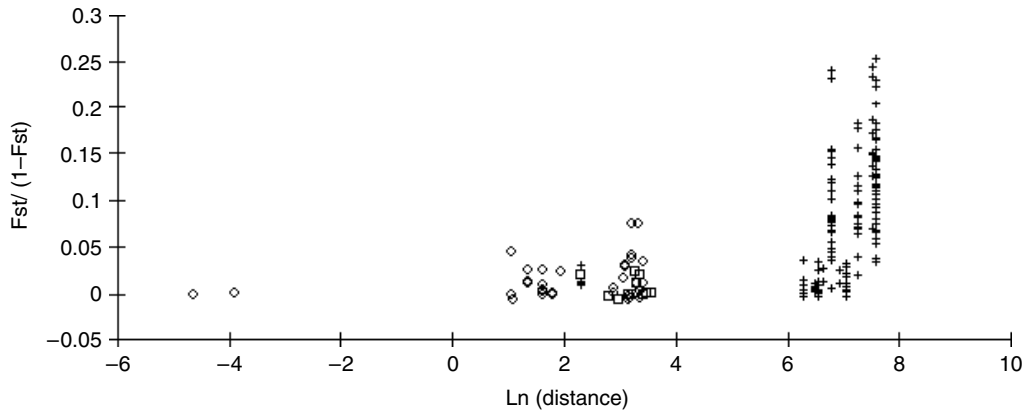


Fig. 2. Relationship between pairwise $F_{st}/(1-F_{st})$ values and the logarithm of geographic distance (in km) for all samples. Circles, squares, triangles and diamonds represent pairs of samples from Guangdong province, Beijing, Hubei province and Henan province, respectively. Pairs from different provinces are indicated with a '+' sign. See text for details.

Genotypic associations between each pair of loci, in each population, were tested using the probability test described by Raymond & Rousset (1995a). For each locus pair, global tests (Fisher's method) were carried out across all populations. Deviations from HW equilibrium were measured using the Fis estimator proposed by Weir & Cockerham (1984). Genotypic differentiation between populations was tested for by calculating an unbiased estimate of the P -value of a log-likelihood (G) based exact test (Goudet *et al.*, 1996). Population differentiation was measured using the F_{st} estimator (Weir & Cockerham, 1984). Differentiation of larger set of populations (here province) was measured and tested using the computer package HierFstat (Goudet, 2005). Isolation by distance was analysed as described by Rousset (1997), by calculating pairwise estimates of $F_{st}/(1-F_{st})$ with respect to the logarithm of geographic distance. Any possible positive relationship was tested with a Mantel test, using the Spearman rank correlation coefficient statistic. Geographical distances between samples were taken as the shortest distance on a map. Calculations were performed using Genepop version 3.4 (Raymond & Rousset, 1995b) and the sequential Bonferroni method (Hochberg, 1988) was used for multiple testing.

Results

Description of polymorphism

A total of 1376 mosquitoes were analysed for the four enzyme systems, among which five putative loci (*Got-1*,

Got-2, *Hk*, *Pgi* and *Pgm*) were revealed. All loci were polymorphic except for *Got-1*, which was not used in the analysis because it was unreadable. Overall, 5245 genotypes were available for analysis. The frequencies of each allele for *Got-2*, *Hk*, *Pgi* and *Pgm* in each sample are shown in table 2.

Statistical independence among loci

Genotypic associations were tested at each pair of loci in each sample. Random association was rejected ($P < 0.05$) in seven out of 97 tests (or 7.2%) and one remained significant when taking into account multiple tests. A global test across populations for each locus pair revealed no pairs with significant values ($P > 0.05$).

Hardy-Weinberg equilibrium

Significant departure from HW equilibrium, due to heterozygote deficiency, was observed in five out of 66 cases (table 2). When the number of tests was taken into account none of the results were significant. For all loci and samples, no significant ($P > 0.45$) heterozygote excess or deficiency was found.

Genetic differentiation

The overall genotypic differentiation found across China was moderate ($F_{st} = 0.059$) and highly significant ($P < 10^{-4}$) (table 3). This genetic variation was partially explained by distance, as a significant ($P < 5.10^{-5}$) increase of differentiation was found with geographic distance, with a slope of

Table 4. Review of population structure studies of the *Culex pipiens* complex.

Geographic area	Subspecies	<i>n</i>	Genetic differentiation	IBD (method)	Distance	Reference
Barcelona (Spain)	m and p	45	$F_{st}=0.067$	–	A	Chevillon <i>et al.</i> (1995c)
Chambery (France)	M	5	$F_{st}>0.15$	N (a)	A	Chevillon <i>et al.</i> (1998)
Chambery (France)	P	9	$F_{st}<0.0013$	N (a)	A	Chevillon <i>et al.</i> (1998)
London (UK)	M	7	$G_{st}=0.15$	N (c)	A	Byrne & Nichols (1998)
London (UK)	P	11	$G_{st}=0.06$	N (c)	A	Byrne & Nichols (1998)
Tunis (Tunisia)	m and p	5	$F_{st}=0.083$	–	A	Krida <i>et al.</i> (1998)
Beijing (China)	pal	5	$F_{st}=0.07$	N (a)	A	Present study
Guangdong (China)	q	9	$F_{st}=0.011$	N (a)	A	Present study
France	m	11	$D=0.039$	–	A	Pasteur (1977)
Madagascar	q and p	9	$F_{st}=0.134$	–	B	Urbanelli <i>et al.</i> (1995)
France	p	44	$D=0.034$	–	B	Pasteur (1977)
Israel	m and p	4	$D=0.011$	–	B	Villani <i>et al.</i> (1986)
Corsica & Sardinia	p	13	$F_{st}=0.048$	N (a)	B	Chevillon <i>et al.</i> (1995a)
Florida (USA)	q	6	$F_{st}=0.057$ $D=0.005$	N (a)	B	Nayar <i>et al.</i> (2003)
Tunisia	p	13	$F_{st}=0.037$	N (a)	B	Ben Cheikh <i>et al.</i> (1998)
Egypt	m and p	9	$D=0.013$	–	C	Villani <i>et al.</i> (1986)
Egypt	p	10	$D=0.013$	–	C	Farid <i>et al.</i> (1991)
France & Spain	p	40	$F_{st}<0.04$	Y (a)*	C	Chevillon <i>et al.</i> (1995b)
West Africa	q	17	$F_{st}=0.04$	Y (a)	D	Chandre (1998)
Vietnam	q	8	$F_{st}=0.06$	Y (a)	D	Pasteur <i>et al.</i> (2001)
Africa	q	5	$D=0.004$	–	D	Urbanelli <i>et al.</i> (1985, 1986)
French Polynesia	q	28	$F_{st}=0.092$	Y (a)	D	Pasteur <i>et al.</i> (1995)
Italy	m and p	20	$D=0.027$	–	D	Urbanelli <i>et al.</i> (1981)
Italy	P	11	$F_{st}=0.009$	Y (a)	D	Silvestrini <i>et al.</i> (1998)
America	q and p	29	–	Y (b)**	D	Cheng <i>et al.</i> (1982)
China	q and pal	20	$F_{st}=0.059$	Y (a)	D	Present study

Subspecies are *C. p. pipiens* (p), *C. p. quinquefasciatus* (q), *C. p. pallens* (pal), *C. p. molestus* (m). The number of populations sampled is indicated (*n*). Isolation by distance (IBD) was tested in each study using a Mantel test between pairwise geographical distance (or its log) and pairwise differentiation. Pairwise differentiation was measured using F_{st} (or $F_{st}/(1-F_{st})$, $(1/F_{st}-1)/4$, or $\log[(1/F_{st}-1)/4]$), G_{st} or D (these three methods are referred to as a, b, or c, respectively). Y or N indicates significant or non-significant IBD, respectively. The geographic distance considered in each study is classified as A (0–100 km), B (100–500 km), C (500–1000 km) or D (> 1000 km). See text for details.

* Reanalysed in Silvestrini *et al.* (1998).

** Reanalysed in Chevillon *et al.* (1995b).

0.018 (fig. 2). In order to investigate the differentiation hierarchically, several groupings were considered.

The intra-province genotypic differentiation within each province was low but significant (Guangdong, $F_{st}=0.011$, $P<0.001$; Beijing, $F_{st}=0.007$, $P<10^{-4}$; Hubei, $F_{st}=0.016$, $P<0.001$; Henan, $F_{st}=0.013$, $P=0.01$, see table 3). In Beijing and Guangdong province, the differentiation did not depend on geographic distances, with isolation by distance not being significant (Beijing, $P>0.20$; Guangdong, $P=0.13$; fig 2). The inter-province differentiation between the five provinces was moderate (hierarchical $F_{st}=0.063$) and highly significant ($P<10^{-3}$), see table 3. A significant isolation by distance ($P=0.016$) was detected with a slope of 0.13.

Discussion

The present study showed that the overall genotypic differentiation across 20 Chinese *C. pipiens* populations was moderate ($F_{st}=0.059$), despite the maximum distance between the populations being about 2000 km and there being isolation by distance at this scale.

We reviewed the literature to compare this result with previous population genetic surveys of this mosquito species in different geographic regions. We found various geographical scales, from a few kilometres (e.g. a city) to the large part of a continent. Therefore, we attributed these distances

to four classes to allow comparisons: 0–100 km (A), 100–500 km (B), 500–1000 km (C) and more than 1000 km (D). Population differentiation was estimated using either D (Nei, 1972), G_{st} (Nei, 1973) or F_{st} (Weir & Cockerham, 1984). These measures do not use the same assumptions (e.g. the genetic distance, D , assumes a genetic independence between the samples, unlike F_{st} , which takes into account a possible gene flow between samples), and are tentatively given in table 4. Isolation by distance (IBD) is more consistent, as it is tested using a Mantel test on pairwise measures of distance and genetic differentiation. As the Mantel test is based on rank, it is not affected by monotonous transformations of pairwise measures. Therefore, the results of the Mantel test remain unchanged if distance is replaced by \log (distance), or if F_{st} is replaced by $F_{st}/(1-F_{st})$ or by $(1/F_{st}-1)/4$. This latter case corresponds to the formula for the number of migrants, N_m , in the infinite island situation (Wright, 1969), and only the sign of the correlation changes. However, when the genetic differentiation is measured using either D or G_{st} , the results are not immediately comparable. It is apparent from table 4 that isolation by distance cannot be detected if the areas considered are less than 500 km apart, but can be definitively detected for areas at least 1000 km wide. These results are independent of the continent considered; there are both small and large scales studies in America, Africa, Europe

and Asia that show contrasting results for IBD. These results also seem to be independent of the subspecies; *C. p. pipiens*, *C. p. quinquefasciatus* and *C. p. pallens* all show an absence of IBD at local scales, and IBD at extended regional scales. There is no equivalent result for *C. p. molestus*, as there have been no large-scale studies for this subspecies. In conclusion, *C. pipiens* requires a considerable distance to display IBD, which appears to be true for most of its distribution ranges. In addition, none of the population genetic studies in table 4 are using highly informative loci (associated with a high mutation rate), such as microsatellites, thus extending the minimum distance at which IBD could be detected in these studies. Detection of IBD is a function of $D\sigma^2$, with σ^2 the migration variance, and D the population density (Rousset, 1997; Leblois *et al.*, 2003). The migration variance has been estimated around 6–7 km in the Montpellier area (Lenormand *et al.*, 1998); and its validity outside this area, including for other climatic and population density conditions, needs to be established. The population density itself is highly variable, and could display very high values (see e.g. Hayes, 1975; Reisen *et al.*, 1992), thus generating values of $D\sigma^2$ outside the range usually considered (Leblois *et al.*, 2003). It is thus presently not possible to extract information from table 4 using the population genetics theory on IBD.

This is the first population genetic survey of *C. pipiens* on an extensive scale in Asia. The south–north sampling encompasses the transition between two subspecies, *C. p. quinquefasciatus* and *C. p. pallens*, in which their relationship is not well characterized. Similar insecticide resistance genes have recently been found in both the Chinese *C. p. pallens* and *C. p. quinquefasciatus* (Cui *et al.*, 2006a,b). Some of these resistance genes are endemic to China, suggesting that their co-occurrence in *pallens* and *quinquefasciatus* is due to gene flow between these two subspecies and not independent or recent introductions from elsewhere. Selected genes (such as insecticide resistance genes) are interesting tools for detecting possible gene flow between two related taxa (e.g. Weill *et al.*, 2000). The co-occurrence of the same insecticide resistance gene in the various subspecies of the *C. pipiens* complex worldwide shows that gene flow is possible (Raymond *et al.*, 1991, 2001). However, these gene categories are not sufficient to quantify the gene flow. The present study was not designed to estimate gene flow between *pallens* and *quinquefasciatus* in China, although we would probably have detected a drastically restricted gene flow. Further studies are required to understand the relationship between *pallens* and *quinquefasciatus* in China, which possibly reflects the hybrid zone between *C. p. quinquefasciatus* and *C. p. pipiens* in America (Cheng *et al.*, 1982).

The extended area that is required to detect IBD suggests that this mosquito migrates over large distances. This is consistent with indirect measures of the distribution of a one-generation migration, which has a standard deviation of 6–7 km (Lenormand *et al.*, 1998). This extensive migration has implications for insecticide resistance monitoring, as any new resistance gene will rapidly spread over a very large geographic area. As *C. pipiens* is a vector of several human pathogenic organisms (e.g. West Nile virus), this large migration distance has also epidemiological implications.

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